THE REACTION BETWEEN AMINO-ACIDS AND CARBOHYDRATES AS A PROBABLE CAUSE OF HUMIN FORMATION

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The study of the black substances obtained when proteins are hydrolyzed in strong acid solution is of great interest at the present time on account of their bearing on the natural melanins and on the quantitative determination of certain amino-acids in proteins. Grindley[1] and his coworkers state that humin nitrogen causes an error in the analysis for amino-acids of common foodstuffs when the Van Slyke amino nitrogen determination is directly applied to them. This view on theoretical grounds was also expressed by Hart and Bentley.[2] It is therefore very important to know more about the structure and mode of formation of these compounds.

Mulder[3] was the first to show that albumins separate flocculi of a brown or black color on being boiled with concentrated hydrochloric or sulfuric acids. Hausmann[4] made similar observations with globin. Samuelly[5] pointed out that the formation of these "artificial melanins" or "melanoidins" might be a secondary reaction between amino-acids and carbohydrates. Maillard[6] conducted experiments along this line and found a

* The work described in this article forms part of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

[1] Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.

[2] Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii, 477.

[3] Mulder, G. J., in Mann, G., Chemistry of the Proteids, London, 1906, 87.

[4] Hausmann, W., *Z. physiol. Chem.*, 1900, xxix, 140.

[5] Samuelly, F., *Beitr. chem. Phys. u. Path.*, 1902, ii, 355.

[6] Maillard, L. C., *Compt. rend. Acad.*, 1912, cliv, 66.

number of them reacted with sugars. His experiments, however, were carried on in aqueous solutions at a very high concentration and temperature and it is doubtful whether under these conditions the reaction is similar to what takes place in the formation of either the "natural" or artificial melanins. It will be shown later in this paper that not all the amino-acids found reactive by Maillard reacted at all at low concentration in water. Gortner and Blish[7] made the important discovery that when tryptophane is boiled with sugar in 22.9 per cent hydrochloric acid solution 86 per cent of its nitrogen is converted into humin nitrogen. They conclude from their experiments that tryptophane alone is responsible for humin formation. Grindley and his coworkers[1] disagree with this conclusion since they found evidence that other amino-acids give the same reaction.

In view of these conflicting statements and in the hope that the study of the reaction between amino-acids and carbohydrates would throw some light on the structure and mode of formation of the humin substances, it was thought worth while to determine: (1) Which amino-acids react with carbohydrates under a given set of conditions. (2) Whether different sugars behave alike toward the same reactive amino-acid. (3) What group of the reactive amino-acids takes part in the reaction.

REVIEW OF THE LITERATURE.

Udránszky[8] and Hoppe-Seyler[9] have shown that when sugar is boiled with acids humin substances are formed, and if boiled in the presence of a nitrogenous material, the humins may also contain nitrogen. Udránszky found, for example, that glucose and urea boiled together in strong hydrochloric acid solution formed humins which contained about 6.73 per cent nitrogen.

Samuelly[5] was the first to study the behavior of humins, or "melanoidins," towards oxidizing and reducing agents. He prepared his "melanoidins" from commercial serum albumin according to Schmiedeberg's[10] method modified by himself. He subjected his product to the action of

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[7] Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630. After this work was completed and sent for publication another article by Gortner on humin formation appeared (*J. Biol. Chem.*, 1916, xxvi, 177). In this article Gortner admits that amino-acids other than tryptophane may be involved in humin formation, which is in harmony with the results reported here.

[8] Udránszky, L. v., *Z. physiol. Chem.*, 1888, xii, 33.

[9] Hoppe-Seyler, F., *Z. physiol. Chem.*, 1889, xiii, 66.

[10] Schmiedeberg, O., *Arch. exp. Path. u. Pharm.*, 1897, xxxix, 1.

HI and PI_3 in a closed tube kept for 8 hours at 200–210°C. Among other products he obtained pyrrol, as detected by the color reaction with pine shavings, and either pyridine or piperidine or some derivative of either one of these bases. Ammonia was also evolved in large amounts. None of the reduction products obtained as above gave indol or skatol on fusion with alkalies, while the humins before reduction gave on similar treatment an unmistakable odor of both. Samuelly also tried reduction with zinc dust in a current of hydrogen. From this treatment he obtained pyridine, pyrrol-like substances, skatol, and small amounts of an aromatic compound of the benzaldehyde series. The same investigator prepared humin substances from some amino-acids and glucose. He[11] heated for 18 hours a mixture of 10 gm. of glucose, 50 cc. water, 15 cc. concentrated HCl (sp. gr. 1.19), and sufficient amount of the different amino-acids so as to have in the solution 0.7 gm. of nitrogen. He tried ammonium chloride, urea, acetamide, glycocoll, aspartic acid, cystine, and tyrosine. In each case he found nitrogen in the melanin. No attempt was made, however, to determine whether the humin nitrogen formation was due to adsorption or to a definite reaction. It is interesting to note that all of the humins so prepared gave off pyrrol on dry distillation with zinc dust, no pyridine, and on fusion with alkalies only the humin prepared from tyrosine produced an odor of indol. Nencki[12] and Berdez[13] obtained similar results with the natural melanins. By alkali fusion these authors obtained from tumor melanin, indol, skatol, volatile fatty acids, hydrocyanic acid, succinic acid, and other unidentified products. Pyrrol was obtained on dry distillation, and after heating the melanin to 300°C. for some time, upon addition of an alkali pyridine was detected.

Gortner and Blish[7] heated pure zein, plus tryptophane, plus carbohydrate in 22.86 per cent HCl, and obtained 16.5 per cent of the total nitrogen of the mixture in the humin form. When tryptophane alone was heated with sugar in acid solution 86.56 per cent of its nitrogen was found in the humins. On the other hand, when histidine plus zein was heated with acid only 0.51 per cent of the total nitrogen was found in the humins. This amount was almost the same as that obtained when zein alone was heated in acid. They did not try, however, heating zein, plus histidine, plus sugar, in acid. Among the conclusions these authors draw from their experiments are the following: (1) The humin nitrogen belongs to "no amino-acids other than tryptophane." (2) "The reaction involved . . . . is probably the condensation of an aldehyde with the —NH group of the tryptophane nucleus." (3) Histidine can be eliminated "as a factor in the formation of humin nitrogen."

Grindley and Slater[1] have tried to apply the Van Slyke amino nitrogen determination directly to the analysis of feedingstuffs. As is to be expected,

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[11] Samuelly, *Beitr. chem. Phys. u. Path.*, 1902, ii, 383.

[12] Nencki, M., and Sieber, N., *Arch. exp. Path. u. Pharm.*, 1887, xxiv, 17.

[13] Berdez, J., and Nencki, M., *Arch. exp. Path. u. Pharm.*, 1886, xx, 346.

on account of the high carbohydrate content of these, the humin fraction in their nitrogen distribution is very high, varying from 3.85 per cent in blood meal to 15.79 per cent in alfalfa hay, expressed as *per cent* of the total nitrogen. In discussing the origin of these humin substances these investigators disagree with the conclusion arrived at by Gortner and Blish, that the humin nitrogen of protein hydrolysis has its origin exclusively in the tryptophane nucleus, since they have obtained "results that clearly indicate that in addition to tryptophane a number of other amino-acids when gently boiled with 20 per cent HCl for 24 to 30 hours in the presence of pure glucose give humin nitrogen. Preliminary experiments show that under the above treatment 4.7 to 6.3 per cent of the total nitrogen of lysine and cystine respectively is separated as humin nitrogen."

Since Bourquelot and Bertrand discovered tyrosinase, this enzyme has received much attention from a great number of investigators. Only that part of the work relating to the action of tyrosinase on the different amino-acids and related substances will be reviewed here.[14]

The effect of tyrosinase on tyrosine is described by Bertrand.[15] A solution of tyrosine to which an extract of tyrosinase is added first becomes red, then inky black, and finally deposits a black precipitate. He proved definitely that atmospheric oxygen is essential to the change by conducting experiments *in vacuo* and in the air. Von Fürth and Schneider[16] used the blood (hemolymph) of the pupæ of a butterfly, *Deiciphila elpenor.* They separated the enzyme from the other substances in the blood by fractional precipitation with ammonium sulfate. It was found to give a yellowish red substance with pyrochatechol; with hydroquinone it gave a red solution, which then became turbid and finally deposited a considerable brownish precipitate. It also acted on adrenalin, giving a dirty brown color. Oxyphenylethylamine became yellowish brown and finally gave an olive-colored precipitate. But tyrosinase has no action on casein itself. The same authors isolated the black substance produced from tyrosine by the tyrosinase of *Deiciphila* pupæ and determined its elementary composition. Below is given a comparison between the percentage composition of this black substance and of tyrosine respectively:

	Black substance (humin) from tyrosine. *per cent*	Tyrosine. *per cent*
C	55.66	59.60
H	4.45	6.08
N	13.74	7.74
O	26.37	26.58

[14] Bourquelot and Bertrand, G., *Bull. Soc. Mycol.*, 1896, xii, 18. A complete list of references up to the time of its publication is found in Kastle's Oxidases, *Bull. Hyg. Lab.*, 59.

[15] Bertrand, G., *Compt. rend. Soc. biol.*, 1896, cxxii, 1215.

[16] Von Fürth, O., and Schneider, H., *Beitr. chem. Phys. u. Path.*, 1901, i, 229.

In the formation of this "artificial melanin" from tyrosine there is an increase in the nitrogen content from 7.74 to 13.74. Such an increase is only conceivable in one of two ways; either there is a breaking up of the tyrosine molecule, or some other nitrogenous substance besides tyrosine takes part in the formation of the melanin. The latter must be the case since tyrosinase, being but a weak oxidizing agent, would be unable to break down the benzene nucleus. The nitrogenous compound that took part in the reaction must evidently have come from the tyrosinase preparation itself. This black product also yields a skatol-like odor on fusion with alkali. In connection with the wide distribution of tyrosinase in both the vegetable and animal kingdom the following is quoted from Kastle's monograph:

"Von Fürth and Schneider are therefore of the opinion that probably wherever melanotic pigments occur in the living tissues of the lower and higher animals they originate as the result of the action of appropriate enzymes on substances of aromatic nature. They point out in this connection that Salkowski and Jacoby have shown independently that tyrosine results from the autolysis of various animal tissues. It would seem likely, therefore, that in the formation of melanotic pigments two ferments are jointly concerned: one, an autolytic ferment capable of splitting off tyrosine or a similar aromatic complex from the protein molecule, and the other tyrosinase, which transforms the tyrosine into melanin."

But one of the most interesting phases of the investigations on tyrosinase is that relating to its effect on the products of protein degradation and related substances. Bertrand and Rosenblatt[17] have found that this enzyme acts equally well upon racemic and *l*-tyrosine. Chodat and Staub[18] discovered that albumoses do not give a red color with tyrosinase but glycyltyrosine anhydride gives such a coloration. In a later article[17] these authors observed that glycine, leucine, and alanine retard the action of tyrosinase; that dipeptides such as tyrosine anhydride, and glycyltyrosine anhydride produce yellow substances which do not become black as does tyrosine itself. When, however, glycine, leucine, or alanine is present, a red coloration similar to that resulting from tyrosine is obtained: glycyltyrosine anhydride with glycine gives a rose color changing to bluish green; with alanine the color is deeper red, with leucine deep brown. But their most striking discovery is that phenylalanine is not acted on by tyrosinase. This, however, acts readily on *p*-cresol, less readily on *m*-cresol, and still less readily on *o*-cresol. In fact these same authors observed that the enzyme acts most readily on the para-homologues of phenol. Amino-acids like glycine increase the rapidity of the action of tyrosinase on *p*-cresol, producing a violet color which ultimately becomes blue. Bertrand undertook to investigate the action of tyrosinase

[17] Bertrand, G., and Rosenblatt, M., *Compt. rend. Soc. biol.*, 1908, cxlvi, 304.

[18] Chodat, R., and Staub, *Arch. Sc. Phys. Nat.*, 1907, xxiii, 265; xxiv, 172.

from wheat bran on compounds analogous to tyrosine and to phenylalanine; that is, compounds with and without the phenolic hydroxyl group. Thus he found phenylalanine, phenylethylamine, phenylmethylamine, phenylaminoacetic acid, phenylpropionic acid, phenylacetic acid, alanine, and glycocoll produced no coloration at all. On the other hand the following compounds with phenolic hydroxyl groups produced coloration as follows:

Tyrosine	Grenadine-red, inky black.
p-Hydroxyphenylethylamine	Grenadine-red, olive-black.
p-Hydroxyphenylmethylamine	Orange-yellow, orange-red, clear maroon.
p-Hydroxyphenylamine	Orange, mahogany-red, brown.
p-Hydroxyphenylpropionic acid	Orange-yellow, grenadine-red, brown.
p-Hydroxybenzoic acid	Rose, orange, yellow.
p-Cresol	Yellow, orange, red.
Phenol	Yellow, orange, red, brown.

He concludes, therefore, that tyrosinase acts only on those compounds containing a phenolic group.

In 1907 Abderhalden and Guggenheim[19] published an interesting article on the effect of tyrosinase from *Russula delica* on tyrosine, tyrosine-containing polypeptides and other related substances. They observed that glycocoll, *d*-alanine, *d*-valine, *l*-proline, *d*-serine, *d*-*l*-isoserine, and *l*-phenylalanine retard the action of tyrosinase on tyrosine only slightly unless present in very large concentrations. The largest concentration used was molar; *l*-aspartic acid and *d*-glutamic acid, however, even when present at a concentration of 0.01 molar retard the action considerably. The same authors found that the enzyme has no effect on diiodotyrosine, *l*-phenylalanine, *l*-proline, or cystine. But *l*- and *d*-tyrosine, homogentisic acid, and tryptophane showed a color change. Particularly interesting was the case of *d*-tryptophane. The authors state that at first they thought that the coloration with tryptophane may be due to traces of tyrosine. They, however used a very pure product. They repeated their experiment but always came to the same result. Furthermore, they tried the effect of tyrosinase on solutions of tryptophane-containing polypeptides and found development of color. They therefore conclude that this coloration must not be ascribed to the presence of traces of tyrosine. Still more interesting is the fact that neither indol nor skatol were found to produce coloration. Abderhalden and Guggenheim in the same article describe the effect of tyrosinase on polypeptides containing tyrosine. The color developed in these cases is modified to some extent by the nature of the amino-acid combined with the tyrosine in the polypeptide. Addition of some amino-acids were also found either to accelerate or to retard the action of tyrosinase on the polypeptide. Thus proline acceler-

[19] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907–08, liv, 331.

ates considerably the action of the oxidase on glycyl-*l*-tyrosine anhydride, while aspartic acid and glutaminic acid retard the action. On the other hand halogen derivatives of the polypeptides were not acted upon by tyrosinase. The same authors also found, as did Bertrand, that tyrosinase acts on phenol, giving a brown color, which was modified by amino-acids. Thus glycocoll plus phenol gave a cochineal color, while proline and phenol gave violet. The authors finally concluded that the amino-acids, when present, apparently take part in the production of the pigment. In a later article[20] these same authors point out that tyrosinase acts on adrenalin with the rapid production of a red color and ultimately dark red flocculi. All three isomers of adrenalin are affected with equal rapidity.

It is to be regretted that in none of the above cited contributions was either arginine, histidine, or lysine tried. It is hoped that this omission will be filled in the near future.

EXPERIMENTAL.

The fact that zein, when boiled with glucose in 22.68 per cent hydrochloric acid solution, increases its humin nitrogen from 0.56 to 1.84 per cent indicates, as Grindley and his coworkers[1] suggested, that other amino-acids besides tryptophane take part in nitrogenous humin formation. Only a small per cent of some of these amino-acids may take part in this formation so that only by working with the individual amino-acids is it possible to determine whether the humin nitrogen was due to a definite reaction or to an adsorption. Again it is only by working under approximately the same set of conditions that it is possible to detect differences in behavior between the different amino-acids. The following procedure was, therefore, adhered to as consistently as practicable.

The amino-acid, plus sugar, plus 50 cc. of water or hydrochloric acid solution of the specified strength was heated for 48 hours in a 300 cc. Kjeldahl flask on a sand bath. The flask was provided with a reflux condenser made from a large test-tube fitted with cork and tubings for a current of cold water. After heating, the digestion mixture was neutralized with the calculated amount of sodium hydroxide solution. The salt thus formed coagulated most of the precipitate that may have existed in a colloidal state in the solution. The mixture was then filtered into 200 cc. graduated flasks and the humin was washed repeatedly

[20] Abderhalden and Guggenheim, *Z. physiol. Chem.*, 1908, lvii, 329.

with boiling water until the flask was filled to the mark. This amount of washing was found to be sufficient to remove almost all of the adsorbed amino-acid which could be removed by this treatment alone. The humin with the filter paper was then Kjeldahled. The filtrate was either Kjeldahled or Van-Slyked or used for both determinations. 25 cc. portions were taken for the Kjeldahl and 10 cc. portions for the Van Slyke determination.

The nitrogen content of the amino-acids was determined either by Kjeldahl's or by Van Slyke's method or by both. The *per cent* of nitrogen was the only thing used to establish the identity and purity of the compounds.

The following amino-acids were furnished by Professor Hart:

Amino-acid.	Nitrogen. Found. *per cent*	Nitrogen. Theoretical. *per cent*
Alanine	15.70	15.75
Cystine	11.2–11.6	11.67
Tyrosine	7.67	7.72
Lysine hydrobromide ($2C_6H_{14}N_2O_2 . HBr . H_2O$)	14.72	14.32
Tryptophane	6.44 (Amino N)	6.86
Phenylalanine*	6.95	6.91

* The phenylalanine was kindly furnished by Dr. T. B. Osborne of New Haven.

The following amino-acids were prepared:

Amino-acid.	Nitrogen. Found. *per cent*	Nitrogen. Theoretical. *per cent*
Leucine, from zein	10.90	10.70
Proline, from zein, also from gelatin	12.20	12.17
	(No amino N)	
Glutaminic acid, from gliadin	7.17	7.61
Arginine (free) from gelatin	29.95	32.20
Amino N	7.70	8.04
Histidine dihydrochloride, from blood	17.25	18.42
Amino N	5.76	6.14

In the preparation of the above amino-acids the directions given in Abderhalden's *Arbeitsmethoden* were followed. The *per cents* of nitrogen found for arginine and histidine respectively were not quite up to the theoretical, but since the amino nitrogen was almost one-fourth of the total in the arginine sample and one-third in the histidine, it was evident that the samples of both

these amino-acids were free from other amino-acids, their low total nitrogen content being due to moisture. The nitrogen determinations of these amino-acids were made on the same day that the experiments on humin formation were started.

Due to the scarcity of material it was found impossible to recrystallize some of the amino-acids in order to obtain as pure a product as could be desired.

The results are shown in the following table. All the experiments were in duplicate and average figures are given.

The results show that neither alanine nor leucine give humin nitrogen. Glutaminic acid when boiled with sugar in 2 per cent acid solution yields some humin nitrogen, but none in 20 per cent acid. Attention is called to the fact that glutaminic acid on heating even at the concentration used seems to form pyrrolidon carboxylic acid readily, as evidenced by the loss of activity of its amino nitrogen in Experiments 13, 14, and 16. Such a formation does not take place in strong acid. Phenylalanine yields about 1.65 per cent of its nitrogen in the humin in 20 per cent acid solution. Proline does not give humin nitrogen with glucose with 20 per cent acid, but seems to react to some extent with xylose and fructose in 4.15 per cent acid solution. Cystine with 20 per cent HCl yields about 3.1 per cent humin nitrogen and the noteworthy fact about this amino-acid is the deeply colored filtrate it produced. The same was observed with the filtrate from the tyrosine-sugar experiments. As much as 15 per cent of tyrosine nitrogen may be converted into humin nitrogen.

The cases of the three hexone bases are particularly interesting. When boiled with sugars in 20 per cent HCl solution all three yield some of their nitrogen as humin nitrogen. Arginine and lysine, with sugar, give more deeply colored filtrates than histidine. If the deep coloration of the filtrate indicates reaction, then it must be stated definitely that in the case of cystine, tyrosine, arginine, and lysine in 20 per cent HCl, the humin nitrogen is due to a reaction and not to an adsorption. Another fact that supports the contention that a definite reaction is responsible for humin formation at least in the case of tyrosine is that phenylalanine gives but little humin nitrogen. If this were a case of adsorption, then there should probably be no differ-

TABLE I.

Record of Results.

Experiment No.	Treatment	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Alanine.								
1	0.089 gm. + 20 per cent HCl	0.0		13.88	13.98	0.10		13.98	Filtrate colorless.
2	0.089 gm. + 0.720 glucose + 20 per cent HCl....	0.0		13.81	13.98	0.17		13.98	" light yellow.
3	0.089 gm. + 0.600 gm. xylose + 20 per cent HCl*								
4	0.089 gm. + 0.720 gm. fructose + 20 per cent HCl.....	0.0		14.00	13.98	−0.02		13.98	" " "
5	0.089 gm. + 2.0 gm. glucose + 20 per cent HCl	0.0		14.01	13.98	−0.03		13.98	" " "
6	0.0445 gm. + 0.720 gm. glucose + 20 per cent HCl	0.0		6.51	6.55	0.04		6.55	" " "
	Leucine.								
7	0.131 gm. + 4.15 per cent HCl.....	0.0		14.36	14.36	0.0		14.36	" colorless.
8	0.131 gm. + 0.720 gm. glucose + 4.15 per cent HCl.....	0.0		14.33	14.36	0.03		14.36	" light yellow.
9	0.131 gm. + 0.720 gm. fructose + 4.15 per cent HCl.....	0.0		14.30	14.36	0.06		14.36	" " "

* Determination lost.

10	0.131 gm. + 0.720 gm. glucose + water	0.0		14.40	14.36	−0.04		14.36	Filtrate light yellow.
11	0.131 gm. + 0.720 gm. glucose + 20 per cent HCl	0.0		14.40	14.36	−0.04		14.36	" " "
	Glutaminic acid.								
12	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	0.10		28.60	28.60	0.00		28.70	" " "
		0.35 per cent							
13	0.400 gm. + 2 gm. glucose + 20 per cent HCl	0.46		22.00	28.24	6.70		28.70	" " "
		1.65 per cent							
14	0.162 gm. + 0.720 gm. glucose + water	0.05		4.47	11.55	7.13		11.60	" colorless.
		0.03 per cent							
15	0.162 gm. + 0.720 gm. glucose + 20 per cent HCl	0.00		11.80	11.60	−0.20		11.60	" light yellow.
16	0.162 gm. + water	0.00		4.58	11.60	7.02		11.60	" colorless.
	Phenylalanine.								
17	0.4 gm. + 2.0 gm. glucose + 20 per cent HCl	0.46		27.50	27.32	−0.18		27.80	" light yellow.
		1.65 per cent							
18	0.4 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00		28.00	27.80	−0.20		27.80	" colorless.
19	0.2015 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00		28.00	27.80	−0.20		27.80	" "

TABLE I—*Continued.*

Experiment No	Treatment	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2)	7 Total N calculated.	Remarks
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Proline.								
20	0.115 gm. + 4.15 per cent HCl	0.00	14.07				14.07 100.1 per cent	14.00	Filtrate almost colorless.
21	0.115 gm. + 0.720 gm. glucose + 4.15 per cent HCl	0.00	14.12				14.12 100.9 per cent	14.00	" light yellow.
22	0.115 gm. + 0.600 gm. xylose + 4.15 per cent HCl	0.53 3.66 per cent	13.10				13.63 97.5 per cent	14.00	" " "
23	0.115 gm. + 0.720 gm. fructose + 4.15 per cent HCl	0.33 2.36 per cent	13.98				14.31 102.0 per cent	14.00	" " "
24	0.380 gm. + 2.0 gm. glucose + 20 per cent HCl	0.00							" " "

25	0.6315 gm. + 2.0 gm. glucose + 20 per cent HCl	0.00							Filtrate light yellow.
	Cystine.								
26	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.25 2.9 per cent							Filtrate yellowish brown.
27	0.400 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00							Filtrate almost colorless.
28	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.36 3.1 per cent	43.20	43.60	43.44	−0.16	44.56 99.4 per cent	44.8	Filtrate yellowish brown.
29	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	1.36 3.1 per cent	43.12	43.60	43.44	−0.16	44.48 99.3 per cent	44.8	Filtrate yellowish brown.
30	0.200 gm. + 2.0 gm. glucose + 0.1 cent HCl	0 00	20.84				20 84 89.5 per cent	23.3	Filtrate light yellow.
31	0.200 gm. + 2.0 gm. proline + 2.0 gm. glucose + 20 per cent HCl	1.61*	45.70				47.31 99.4 per cent	47.70	Filtrate yellowish brown.

*3.38 per cent of total N or 6.9 per cent of cystine N.

TABLE I—*Continued.*

Experiment No.	Treatment	1 Humin N (Kjeldahl)	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke)	4 Calculated amino N in filtrate.	5 Amino-N difference	6 Total N found (column 1 + column 2).	7 Total N calculated	Remarks.
		mg.	*mg.*	*mg*	*mg.*	*mg.*	*mg*	*mg*	
	Tyrosine.								
32	0.1586 gm. + 20 per cent HCl	0.00		12.22	12.22			12.22	Filtrate brownish.
33	0.1586 gm. + 0.720 gm. glucose + 2.5 per cent HCl	0.20		12.01	12.02	0.01		12.22	" yellowish brown.
34	0.1586 gm. + 0.720 gm. glucose + 20 per cent HCl.	0.81		11.40	11.38	−0.02		12.22	" brownish.
35	0.1586 gm. + 2.0 gm. glucose + 20 per cent HCl	1.33		10.62	10.89	0.27		12.22	" "
36	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.64		26.00	26.06	0.06		30.70	" "
	Arginine.								
37	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	2.31 1.97 per cent	115.00	28.20	28.90	0.70	117.31 98.1 per cent	119.5	" yellowish brown.
38	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	2.77 2.33 per cent	115.50	28.70	28.95	0.25	118.27 99.0 per cent	119.5	" "
39	0.400 gm. + 2.0 gm. glucose + water	0.35 0.29 per cent	115.00	21.70	28.90	7.20	115.35 96.2 per cent	119.5	" brownish carmine.

	Lysine.								
40	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.54 2.62 per cent	57.5	57.50	57.50	0.00	58.94 100.5 per cent	58.90	Filtrate yellowish brown.
41	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.47 2.50 per cent	57.4	57.00	57.40	0.40	58.77 99.9 per cent	58.90	" " "
42	0.2 gm. + 1.0 gm. glucose + water.	0.28 0.48 per cent	28.3	23.70	28.60	4.90	28.58 98.0 per cent	29.20	" carmine.
	Histidine.								
43	0.1915 gm. + 20 per cent HCl			14.00	14.00			42.00	" light yellow.
44	0.1915 gm. + 0.360 gm. glucose + 4.15 per cent HCl	0.61 1.45 per cent		12.19	13.80	1.61		42.00	" " "
45	0.1915 gm. + 0.720 gm. glucose + 20 per cent HCl	0.77 1.84 per cent		12.81	13.74	0.93		42.00	" " "
46	0.1915 gm. + 0.600 gm. xylose + 4.15 per cent HCl	1.08 2.58 per cent		12.38	13.62	1.24		42.00	" " "

TABLE I—*Concluded.*

Experiment No.	Treatment	1 Humin N Kjeldahl	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke)	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	
47	0.1915 gm. + 0.600 gm. xylose + water	0.68 1.62 per cent		11.53	13.80	2.27		42.0	Filtrate yellowish brown.
48	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	0.80 1.16 per cent	68.00	23.20	22.70	−0.50	68.80 99.8 per cent	69.0	" light yellow.
49	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	0.77 1.12 per cent	67.20	23.40	22.40	−1.00	67.90 98.4 per cent	69.0	" " "
50	0.200 gm. + 1.0 gm. glucose + water	0.00	35.40	11.68	11.80	0.12	35.40 100.0 per cent	35.4	" " "
51	0.200 gm. + 1.0 gm. fructose + water	0.21 0.32 per cent	33.30	8.70	11.10	2.30	33.51 94.6 per cent	35.4	" deep brown.
	Tryptophane.								
52	0.1514 gm. + 1.0 gm. glucose + 20 per cent HCl.	13.82 71.0 per cent	5.44	2.73	2.72	−0.01	19.26 98.8 per cent	19.50	" almost colorless.

ence in behavior between tyrosine and phenylalanine. In aqueous or very weak acid solution arginine, histidine, and lysine evidently react with sugar as indicated by the highly colored solutions produced and by the loss of activity of a large fraction of their amino nitrogen. Thus, when arginine plus glucose was boiled in water there was a very deep coloration of the solution (Experiment 39), and at least 25 per cent of the amino nitrogen became inactive towards nitrous acid. Lysine behaved similarly (Experiment 42), 17 per cent of the amino nitrogen becoming inactive towards nitrous acid. Histidine acted likewise (Experiments 47 and 51), 16.2 per cent of its amino nitrogen becoming inactive. These facts show that in the cases of histidine and arginine the α-amino nitrogen takes part in the reaction. In the case of lysine it is difficult to establish which amino group is reactive, since at the time the amino nitrogen in the filtrate was determined the temperature in the laboratory was about 35°C. and at this temperature it was found that both the α- and the ϵ-amino group of lysine react with nitrous acid in 5 minutes, as may be seen in the amino nitrogen determination of the filtrate (Experiments 40 to 42). It is to be noted that in these cases some loss of nitrogen also took place. It may be that during the reaction some ammonia was given off.

The result with tryptophane is in agreement with the work of Gortner and Blish[7] in that a greater portion of the tryptophane nitrogen is converted into humin. The strength of the acid used here and the different procedure followed may account for the difference in the *per cent* of tryptophane nitrogen found in the humin which according to the above named authors was 86 per cent while in these experiments only about 71 per cent was obtained. Due to a lack of material it was impossible to repeat the experiment with tryptophane.

In order to determine which atomic groupings in tyrosine, cystine, and tryptophane were responsible for humin formation, the humin from each one of these amino-acids was dissolved in 0.1 N alkali and Van-Slyked. It was believed that if the amino groups in this humin remained intact they should still give the nitrous acid reaction. The results are as follows:

	Humin nitrogen. mg.	Reactive with HNO_2. mg.
Tyrosine	2.360	2.45
Cystine	0.974	0.88
Tryptophane	13.820	1.90

From these results it must be concluded that in the case of tyrosine and cystine it was not the amino group that reacted with sugar to form humin but some other group, probably the (OH) in tyrosine and the (S-S) in the case of the cystine. If this were the case, then the cystine would presumably undergo reduction before reacting with the sugar.

In order to determine whether, as Gortner and Blish suggested, the furfurol obtained from sugar was responsible for the reaction, Experiments 54, 55, and 56 were performed as follows:

Experiment No.		Humin N. mg.	Per cent of total N.
54	0.2 gm. cystine + 2 cc. furfurol + 20 per cent HCl	7.00	32.0
55	0.2 gm. tyrosine + 2 cc. furfurol + 20 per cent HCl	8.40	55.0
56	0.2 gm. arginine + 2 cc. furfurol + 20 per cent HCl	12.75	21.5

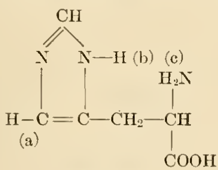
These results tend to show that the furfurol formed from sugars under the influence of acids may to a great extent be responsible for humin formation.

As to the effect of the different sugars on the reactive amino-acid, Experiments 20, 21, 22, 29, 38, and 46 show that xylose and fructose give higher results than glucose as a rule. This is to be expected, if it is admitted that furfurol or some other simple aldehyde is the active substance in these reactions.

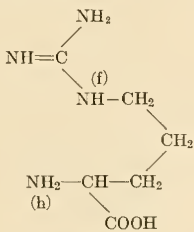
DISCUSSION OF RESULTS.

Some evidence is given which shows that the α-amino groups of arginine, histidine, and tryptophane take part in the reaction with sugars. On the other hand, the α-amino groups of alanine and leucine are unable to give the same reaction. Glutaminic acid and phenylalanine, although giving some humin nitrogen, likewise furnish no indication of reaction. At least for the present it may be admitted that the humin nitrogen in these cases—

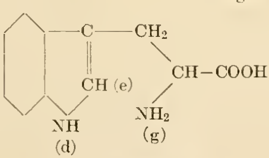
glutaminic acid and phenylalanine—was due to adsorption and not to a reaction. It was also shown that in tyrosine the reactive group is presumably the (OH) and surely not the α-NH_2. In cystine, as shown above, the α-amino group remained intact, so that presumably the reaction was with the mercaptan group. The question may then be asked: Why are the α-NH_2 groups of arginine, histidine and tryptophane more reactive than those of the other amino-acids? An attempt to explain this difference in behavior of the amino-acids towards carbohydrates, based on the present work and on some of the contributions reviewed in the first part of this paper, is here offered. It is generally stated that the properties of a compound are functions of its structure. It is, therefore, to the structure of these amino-acids that we must look for an explanation of their different behavior towards carbohydrates. The structural formulas of histidine, tryptophane, and arginine are given below.



Histidine.



Arginine.



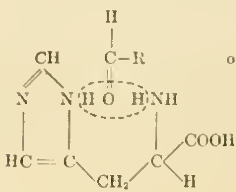
Tryptophane.

Several investigators have advanced the idea that humin formation is dependent on the presence of labile hydrogen in the amino-acid molecule (Samuelly, Grindley and Slater, etc.). Evidently judging from the results of the present work the two hydrogens of the α-amino groups of alanine and leucine are not

labile enough to give condensation products with carbohydrates at least under the conditions of these experiments.

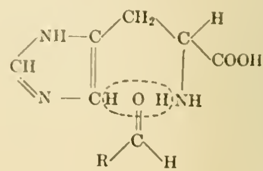
In histidine, arginine, and tryptophane, however, there are other labile hydrogens (a, b, c, d, e, f). The positions of these labile hydrogens with respect to the α-amino group are very favorable for ring formation. The reaction with a carbohydrate or furfurol may very well be thought of as taking place as follows:

Histidine.



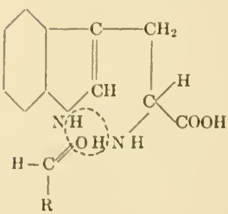
I

or



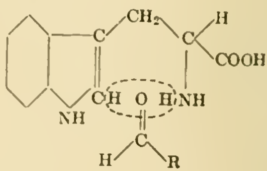
II

Tryptophane.



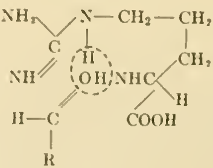
III

or



IV

Arginine



V

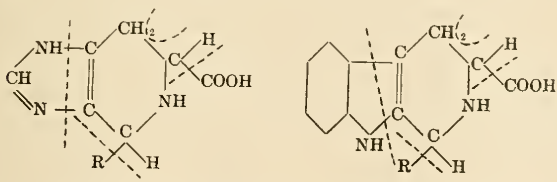
The following facts tend to support the idea of ring formation:

1. The intense color of the products.

2. Miss Homer[21] in her work on the condensation products of tryptophane with aldehydes, speaking of the action of glyoxal on this amino-acid, states:

> "Taking into consideration the necessity of the presence of an oxidizing agent and also the fact that the substance produced is intensely colored it is highly probable that in this reaction, besides the simple aldehyde condensation . . . . there has also been elimination of hydrogen accompanied by complex ring formation."

3. The fact that pyridine was obtained by Samuelly from his "melanoidins," was at one time used as an argument to indicate that a pyridine nucleus was found in proteins. This idea has been disposed of by Emil Fischer's work on proteins, but the fact remains that pyridine is found in the humin formed from proteins. This occurrence may be explained by Reactions II and IV thus:



4. The action of tyrosinase on tyrosine tends to support the idea of ring formation. Tyrosinase produces coloration with tryptophane but not with indol, skatol, or glycocoll. Therefore, the formation of the highly colored product requires the peculiar structure of tryptophane. This formation may be considered as taking place in the manner described above.

The differences in behavior between histidine, arginine, and tryptophane may again be referred back to the differences in their structure. Tryptophane being already a complex compound with a benzene and a pyrrol ring may form an insoluble four-ringed compound with furfurol, which is extremely resist-

[21] Homer, A., *Biochem. J.*, 1913, vii, 111.

ant to the action of acid. This will explain why tryptophane is converted into humin almost quantitatively. On the other hand furfurol may form with histidine and arginine products which are still more or less soluble and in the presence of strong acid may be hydrolyzed back to the free amino-acids. Thus as in the case of glutaminic acid, no formation of a ring compound takes place in strong hydrochloric acid solution. This view will explain why in weak acid or in aqueous solution both histidine and arginine react more readily to form colored products than in strong acid solution.

It is not claimed that the reaction given above gives the actual structure of the melanin molecule, since no evidence is available to indicate what happens to the rest of the molecule of the amino-acids during the reaction with sugars. This theory on humin formation is given here in the hope that it may serve as a guide for future work on the structure of these compounds.

No evidence was found in the present work to explain Samuelly's finding that when the humin obtained from sugar plus tyrosine was fused with alkalies, an odor of indol was obtained. It might have been possible that the tyrosine used contained traces of tryptophane which would explain the production of indol. Likewise the fact that pyrrol was obtained from his "melanoidins" can be traced back to the presence of the tryptophane nucleus in them.

Almost all of the experiments recorded in this paper were done with single amino-acids. There was found evidence (Experiment 31) to show that the reaction would be different, at least in the case of cystine and tyrosine, if other amino-acids were present in the reaction mixture with sugar. If cystine and proline were boiled together in the presence of glucose and 20 per cent HCl a larger amount of cystine nitrogen disappeared in humin formation than when cystine was boiled alone. The same was true when tyrosine and proline were boiled together. It would, therefore, be interesting to study the behavior of mixtures of different amino-acids when boiled with sugars, both in acid and aqueous solutions. Abderhalden and Guggenheim[19] working with tyrosinase along this same line already concluded that other amino-acids, when present, apparently take part in the production of the pigment.

CONCLUSIONS.

1. Alanine, leucine, phenylalanine, and glutaminic acid may be eliminated as important factors in humin formation, when subjected to the treatment used in these experiments. Proline, however, under certain conditions may be involved in humin formation.

2. The following amino-acids were responsible for humin formation, and in digestions, with 20 per cent HCl plus sugar, the proportion of their nitrogen disappearing was: Tyrosine, 15.0; cystine, 3.1; arginine, 2.33; lysine, 2.62; histidine, 1.84; tryptophane, 71.0 per cent.

3. Xylose and fructose were as a rule more reactive than glucose.

4. Arginine, histidine, and lysine reacted with sugars more readily in weak acid or aqueous, than in strong acid solutions.

5. Arginine, histidine, and tryptophane reacted with loss in reactivity of their amino nitrogen towards nitrous acid, but tyrosine and cystine reacted without any such loss.

6. A possible mode of reaction is suggested.

It is with pleasure that the writer acknowledges his obligation to Professor E. B. Hart, Chief of this Department, for giving him this problem, and for his many valuable suggestions during its execution.

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